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High-throughput screening for gene libraries expressing carbohydrate hydrolase activity

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Abstract

A simple and fast method is described allowing screening of large number of *Escherichia coli* clones (4000 per day) for the presence of functional or improved carbohydrate hydrolase enzymes. The procedure is relatively cheap and has the advantage that carbohydrate degrading activity can be directly measured using liquid cultures grown in microtiter plates without the need of separation or purification steps.

Introduction

There is a large variety of carbohydrate structures paralleled by a large number of enzymes involved in carbohydrate processing. Glycoside hydrolases are such enzymes (P.M. Coutinho & B. Henrissat 1999, http://afmb.cnrs-mrs.fr/CAZY/). They play an important role in the carbon and energy supply of many organisms as they can degrade (e.g. hydrolyse) carbohydrate polymers to oligosaccharides that are suitable for uptake by organisms. To identify new, or to improve existing glycoside hydrolase enzymes, simple and effective screening methods are needed, to evaluate relatively large (10^4) genomic DNA, cDNA, environmental DNA, or mutant gene libraries for glycoside hydrolase activities. A good method for this is to screen for the formation of reducing sugars, as hydrolysis of the glycosidic bonds in carbohydrates increases the number of reducing sugar ends. Although several methods are available for this (Somogyi 1952, Nelson 1944, Bernfeld 1955, Green *et al*. 1989, Park & Johnson 1949), these methods have not been adapted for evaluating large numbers of liquid cultures for the presence of such enzymes. To develop such an assay, we adapted the Nelson–Somogyi method for determination of reducing sugars (Somogyi 1952, Nelson 1944). As an example we used an error-prone PCR mutant library of cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* in a screen for CGTase mutants displaying an increased hydrolytic activity. The main activity of CGTases is the formation of circular oligosaccharides (called cyclodextrins) from starch (van der Veen *et al*. 2000). CGTases also display a low hydrolytic activity on starch; *B. circulans* CGTase has k_{cat} values of 338 s^{-1} and 3.8 s^{-1} for the cyclization and hydrolysis reactions, respectively. Only this hydrolytic activity increases the number of reducing sugar ends. With the method described we were able to increase the hydrolytic activity of *B. circulans* CGTase 90-fold.

Materials and methods

Strains, plasmids and growth conditions. Escherichia coli strain MC1061 carrying plasmid pDP66k (encoding the *Bacillus circulans* CGTase) was grown on LB agar plates or in 200 μl LB medium in 96-well microtiter plates (polystyrene) at 37 °C with continuous shaking at 750 rpm. Transformation of *E. coli* was done using $CaCl_2$ competent cells. As substrate for CGTase we used soluble starch.

Screenings procedure. Screenings procedure is shown schematically in Figure 1. *E. coli* clones carrying plas-

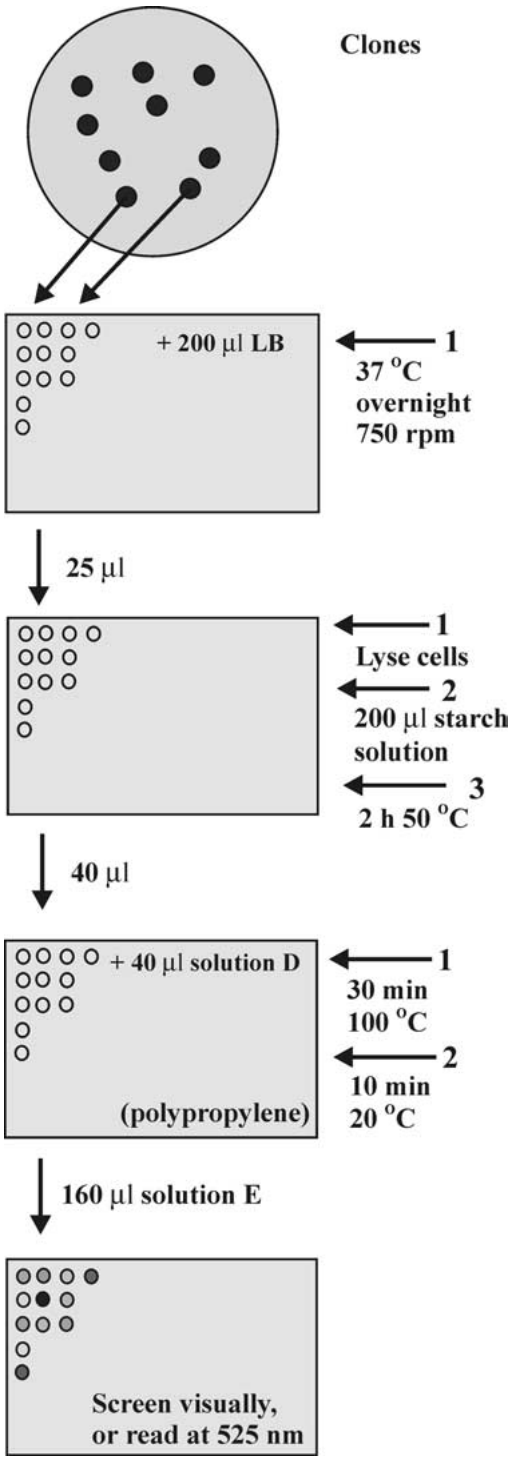

Fig. 1. Schematic representation of the screenings procedure for assaying clones for the presence of carbohydrate hydrolyzing enzymes.

mid pDP66k were transferred to 200 μl LB medium in 96-well microtiter plates either by hand or using the colony picking robot Q-pix (Genetix, New Milton Hamsphire, UK) and the microtiter plates were incubated overnight. Subsequently 25 μl of each culture was transferred to a second microtiter plate (polystyrene) containing 10 μl bacterial protein extraction reagent (Pierce, Rockford, IL) per well. This reagent lysis cells to release the proteins, without denaturating the proteins, allowing activity measurements of enzymes located within cells. After addition of 200 μl 1% (w/v) soluble starch in 10 mM sodium citrate buffer (pH 6) the microtiter plates were incubated in an oven at 50 °C for 2 h. Forty μl of each reaction was added to 40 μl solution D (2.5 g Na_2CO_3, 2.5 g KNa tartrate and 20 g Na_2SO_4 in 100 ml water plus 0.6 g $CuSO_4 \cdot 5H_2O$ and 4 μl 96% (w/v) sulfuric acid in 4 ml water) in polypropylene microtiter plates (Nalge Nunc International, Roskilde, Denmark). After sealing with a polypropylene lid the plates were incubated in an oven at 100 °C for 30 min (polypropylene microtiter plates were used as this material allows heating to 100 °C). After cooling to room temperature on table for 10 min, 160 μl of solution E [1.56 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.31 ml 96% sulfuric acid and 0.19 g $Na_2HAsO_4 \cdot 7H_2O$ in 100 ml water] was added allowing color development. A concentration of 0.25 mM reducing sugars is easily visible, increasing the absorbance from 0.05 to 0.1 at 525 nm. The microtiter plates were screened visually; increasing amounts of reducing sugars give darker colors. Otherwise, the absorbency was measured at 525 nm after pipetting the assay solution into a polystyrene plate (polypropylene plates are not transparent).

Results and discussion

The high-throughput screenings procedure for carbohydrate hydrolase activity was tested using several available site-directed mutants of CGTases with different hydrolytic activities and using starch as substrate. *E. coli* clones expressing mutant CGTases with a higher hydrolytic activity produced more reducing sugars, as indicated by the formation of a dark green color in the assay (Figure 2). In contrast, *E. coli* clones expressing a catalytic dead CGTase mutant (D229A; Knegtel *et al.* 1995), producing no reducing sugars, resulted in light green wells, compared to clones expressing wild-type CGTase (Figure 2). Note that the formation of cyclodextrins (by CGTase) does not in-

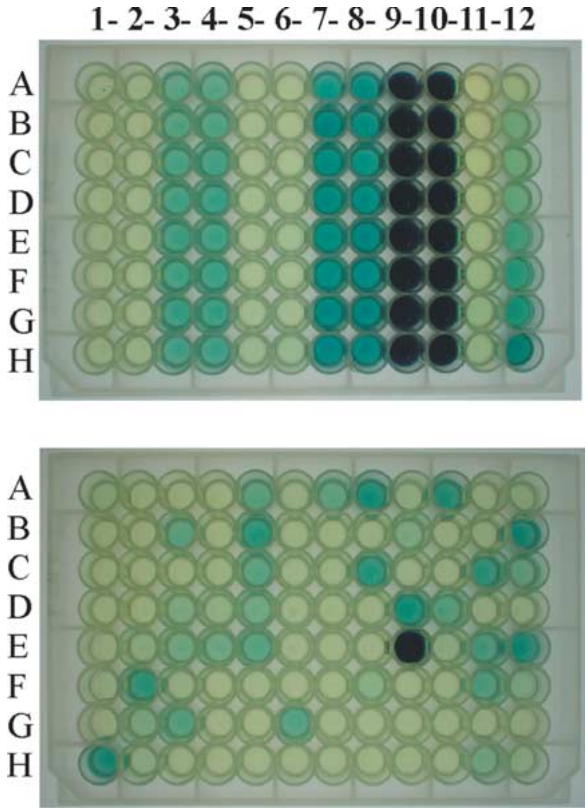

Fig. 2. Detection of starch hydrolysis activity in culture lysates of (mutant) CGTase expressing *E. coli* cells. The upper plate shows the controls: lanes 1–2, *E. coli* without plasmid; 3–4, *E. coli*/pDP66k (wild-type CGTase); 5–6, *E. coli*/pDP66k/D229A (catalytic dead mutant; Knegtel *et al*. 1995); 7–8, *E. coli*/pDP66k/F259N (mutant with increased hydrolytic activity; van der Veen *et al*. 2001); 9–10, *E. coli*/pDP66k/F21L/N94S/A230V (mutant isolated in the screening; Leemhuis *et al*. 2003); 11–12, calibration, 0-0.03-0.07-0.1-0.13-0.17-0.2-0.25-0.3-0.35-0.4-0.5-0.6-0.7-0.8-1 mM maltose. The lower plate shows an example of the screening for mutant CGTases with increased hydrolytic activity. A plate is shown that contains one clone (well E9) with a strongly increased hydrolytic activity.

crease the number of reducing sugar ends. Thus, the methods seems useful for screenings purposes.

To test the procedure in a screening, we used this approach to identify CGTase mutants with increased starch hydrolyzing activity in an error-prone PCR mutant library of *B. circulans* CGTase (Leemhuis *et al*. 2003). An example of this screening is shown in Figure 2. Several mutant CGTase displayed a strongly enhanced starch hydrolyzing activity and the best performing mutant had a 90-fold higher hydrolytic activity than the wild-type enzyme; the k_{cat} increased from 3.8 to 340 s^{-1}. This changed CGTase from a cyclodextrin producing enzyme into a starch hydrolase. The biochemical characteristics and the explanation for the increased hydrolytic activity are described elsewhere (Leemhuis *et al*. 2003). With the described method, 12 000 individual clones could easily be assayed for starch hydrolyzing activity within one week. Day 1 transformation to *E. coli*, day 2 transfer of clones to liquid growth medium, days 3–5 assaying of 4000 liquid cultures each day; all days were less than 8 h work. Thus, the high-throughput screenings method is useful for the identification of mutant CGTases with increased starch hydrolyzing activity.

Conclusions

A fast and simple procedure is described for the identification of *B. circulans* CGTase mutants with increased hydrolytic activity. The procedure has as advantage that it could be applicable to all screening protocols for clones expressing carbohydrate acting enzymes that increase (or decrease) the reducing sugar content. Moreover, the method is compatible with various types of carbohydrates.

Acknowledgements

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